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A Novel Presynaptic Inhibitory Mechanism Underlies Paired Pulse Depression at a Fast Central Synapse

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Summary

Several distinct mechanisms may cause synaptic depression, a common form of short–term synaptic plasticity. These include postsynaptic receptor desensitization, presynaptic depletion of releasable vesicles, or other presynaptic mechanisms depressing vesicle release. At the endbulb of Held, a fast central calyceal synapse in the auditory pathway, cyclothiazide (CTZ) abolished marked paired pulse depression (PPD) by acting presynaptically to enhance transmitter release, rather than by blocking postsynaptic receptor desensitization. PPD and its response to CTZ were not altered by prior depletion of the releasable vesicle pool but were blocked by lowering external calcium concentration, while raising external calcium enhanced PPD. We conclude that a major component of PPD at the endbulb is due to a novel, transient depression of release, which is dependent on the level of presynaptic calcium entry and is CTZ sensitive.

Introduction

Synaptic transmission is subject to transient changes in efficacy on the millisecond to second time scale (Zucker, 1989, 1996; O'Donovan and Rinzel, 1997). Depression of synaptic responses following prior activity is especially prominent at central synapses where evoked synaptic potentials have a high quantal content, such as in the central auditory pathway (Trussell et al., 1993; Barnes-Davies and Forsythe, 1995; Isaacson and Walmsley, 1996; Von Gersdorff et al., 1997; Wang and Kaczmarek, 1998) and cerebellum (Dittman and Regehr, 1998; Hashimoto and Kano, 1998), but can also be seen at synapses with low quantal content (Thomson et al., 1993; Markram and Tsodyks, 1996; Varela et al., 1997; Dobrunz and Stevens, 1997).

Several forms of short-term depression of synaptic strength, due to a number of distinct mechanisms, may exist at different synapses (Dobrunz and Stevens, 1997). Typically, synaptic depression is sensitive to conditions that change release probability, implying that depression is governed presynaptically; lowering release probability decreases depression and may convert it to facilitation, while increasing release probability increases depression (Zucker, 1989; Dobrunz and Stevens, 1997).

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Early studies of synaptic depression proposed that it was due to depletion of an available pool of releasable vesicles, with a consequent reduction in release probability while this pool was replenished (Liley and North, 1953; Thies, 1965; Kusano and Landau, 1975), a hypothesis that still has considerable support today (Stevens and Wang, 1995; Dobrunz and Stevens, 1997; Von Gersdorff et al., 1997; Dittman and Regehr, 1998; Wang and Kaczmarek, 1998). However, release probability may be decreased by presynaptic mechanisms other than depletion (Dobrunz and Stevens, 1997), such as calcium-dependent adaptation of transmitter release (Hsu et al., 1996). At some synapses, postsynaptic receptor desensitization, which can also be dependent on the number of quanta released into the synaptic cleft, may underlie synaptic depression (Trussell et al., 1993; Otis et al., 1996a; Westbrook and Jones, 1996).

The response to paired stimuli at the rat endbulb of Held, a calyceal synapse between cochlear nerve fibers and the soma of bushy cells in the anteroventral cochlear nucleus, shows marked paired pulse depression (PPD) (Isaacson and Walmsley, 1996). We have investigated the mechanism of this synaptic depression at interstimulus intervals (ISIs) of less than 50–80 ms. Cyclothiazide (CTZ), a drug that has both pre- and postsynaptic actions at glutamatergic synapses (Vyklicky et al., 1991; Yamada and Tang, 1993; Diamond and Jahr, 1995; Isaacson and Walmsley, 1996), selectively abolished the marked depression of the second synaptic response at these brief ISIs. Our results indicate that this CTZ-sensitive depression is due to a novel inhibitory mechanism, which is dependent on the level of presynaptic calcium entry in response to the first stimulus and is not due to postsynaptic receptor desensitization nor to presynaptic depletion of releasable synaptic vesicles.

Results

Evoked AMPA EPSCs Show Paired Pulse Depression

Short-term depression at other synapses typically shows several components at various time scales (Von Gersdorff et al., 1997; Dittman and Regehr, 1998). We investigated the time course of PPD at the endbulb synapse, where PPD of EPSCs mediated by α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptors has been previously demonstrated at short ISIs (Isaacson and Walmsley, 1996).

Single fiber-evoked EPSCs, at a holding potential of −70 mV or more negative to isolate AMPA responses, showed depression of the amplitude of the second EPSC when two stimuli were given within 150 ms of each other in all neurons recorded ($n = 30$). The amplitude of the first EPSC remained stable for pairs of stimuli given at 0.33–0.1 Hz, indicating that full recovery of evoked synaptic release occurred within 3 s (Von Gersdorff et al., 1997). Typical responses to pairs of stimuli at different ISIs in control conditions are illustrated in Figure 1A. As shown in Figure 1C, at the minimum ISI tested (5 ms),

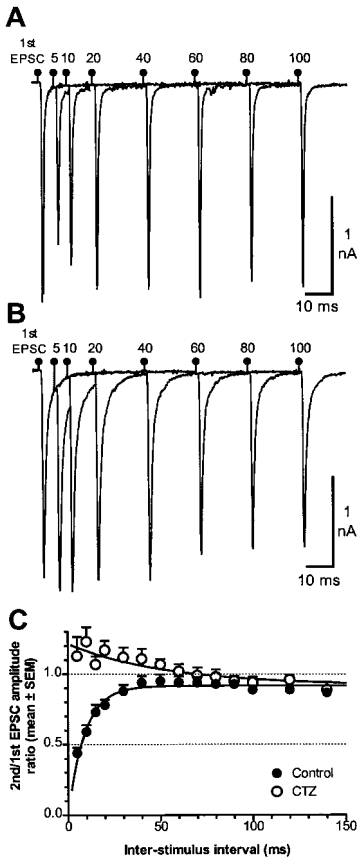

Figure 1. Time Course of Paired Pulse Depression (PPD) of AMPA EPSCs at −70 mV

(A) An example of PPD of AMPA EPSCs. First stimulus (closed circle labeled first EPSC) evokes a large amplitude EPSC; the second stimulus (marked with closed circle) evokes an EPSC whose amplitude depends on the interstimulus interval (ISI; given in ms next to each stimulus marker). Each trace in (A) and (B) is an average of five trials at the same ISI.

(B) Bath application of cyclothiazide (CTZ, 50 μM) slows the decay of evoked EPSCs and abolishes PPD in the same cell.

(C) Mean PPD of AMPA EPSCs at −70 mV and the effect of CTZ on PPD. Each data point is the mean ratio of the second EPSC peak amplitude divided by the first EPSC peak amplitude for different ISIs in several cells (n = 30–24 cells for each control point, n = 5 for responses in CTZ). The best fit for the two data sets to a single exponential function is shown.

the mean ratio of the amplitudes of the second to the first EPSCs was 0.44 ± 0.04 (n = 30). As the ISI increased, the mean EPSC ratio increased until it reached a plateau value of approximately 0.9 at ISIs longer than 50 ms. The mean time course of PPD at −70 mV was best fitted by a single exponential function with a time constant of 9.4 ± 0.6 ms and plateau value of 0.92 ± 0.01 (Figure 1C).

The large degree of PPD at short ISIs and its rapid decrease indicates that PPD is unlikely to be due to release-dependent activation of metabotropic glutamate autoreceptors (Barnes-Davies and Forsythe, 1995; Takahashi et al., 1996; Von Gersdorff et al., 1997) or other presynaptic receptors inhibiting transmitter release, such as $GABA_B$ or adenosine receptors (Hashimoto and Kano, 1998), as activation of these G protein–coupled receptors typically requires more than 50 ms to produce a maximal response (Hille, 1992).

Paired Pulse Depression Is Blocked by Cyclothiazide

Previous work has shown that CTZ, a drug known to block postsynaptic desensitization of AMPA receptors (Vyklicky et al., 1991; Yamada and Tang, 1993), reduces PPD at the endbulb-bushy cell synapse at short ISIs (Isaacson and Walmsley, 1996). We investigated the effects of CTZ at ISIs up to 150 ms and found that CTZ (50 μM) only abolished PPD at ISIs up to approximately 50–80 ms, during the period in which the EPSC ratio was increasing in control recordings; it had no significant effect on PPD at ISIs where the EPSC ratio had reached plateau levels (Figures 1B, 1C, and 2B). The mean EPSC ratio at an ISI of 5 ms in CTZ was 1.13 ± 0.13 (Figure 1C; $n = 5$, $p < 0.0001$, compared with control PPD). This early phase of PPD was thus termed CTZ-sensitive PPD and was further investigated here.

Strontium Enhances Paired Pulse Depression

The effect of CTZ on PPD suggested that postsynaptic desensitization of AMPA receptors might underlie PPD at the endbulb synapse, as has been shown for PPD at the homologous auditory synapse between cochlear nerve fibers and magnocellularis neurons in the chick (Trussell et al., 1993; Otis et al., 1996b). If this were the case, then conditions that alter short-term plasticity by changing presynaptic release probability should be relatively ineffective at altering PPD. We therefore examined the effects of adding extracellular Sr^{2+}, a divalent cation that enhances short-term plasticity presynaptically, without substantial suppression of evoked synaptic release when the external Ca^{2+} level is kept constant (Goda and Stevens, 1994; Van der Kloot and Molgó, 1994; Abdul-Ghani et al., 1996), on PPD.

Addition of 4–10 mM Sr^{2+} (n = 9) to normal ACSF resulted in a decrease in the amplitude of the first EPSC to a mean of 0.79 ± 0.08 of control amplitude (Figures 2A and 2B, $p = 0.026$, one sample t test). This decrease in the first EPSC amplitude, possibly due to Sr^{2+} competing with Ca^{2+} for entry into the presynaptic terminal via voltage-gated Ca^{2+} channels (Lansman et al., 1986), should decrease PPD if it were due to postsynaptic desensitization of AMPA receptors or presynaptic vesicle depletion, since fewer vesicles and less glutamate are released. Contrary to this expectation, the addition of 4 mM Sr^{2+} (n = 5 neurons) increased PPD (mean EPSC ratio at 5 ms ISI = 0.31 ± 0.08, $p = 0.07$, paired t test, compared with control value of 0.48 ± 0.11). Strontium also altered the time course of PPD, slowing the rate of PPD recovery and enhancing the degree of PPD during the plateau phase (Figure 2C). The best fit to the mean EPSC ratio data was a single exponential function with a time constant of 25 ± 2 ms and plateau value of 0.69 ± 0.02 (Figure 2C). These results indicate that PPD can be substantially enhanced by presynaptic effects of Sr^{2+} and suggest that presynaptic Ca^{2+} entry may play a role in PPD.

It is possible that Sr^{2+} might enhance PPD by adding a presynaptically governed component with a slower time course to existing postsynaptic desensitization.

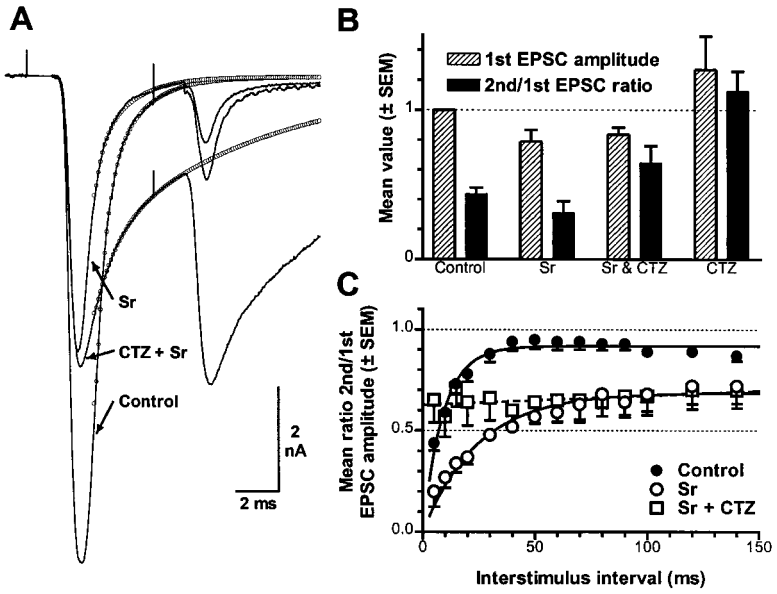

Figure 2. Strontium Increases PPD but Decreases First EPSC Amplitude

(A) An example of paired pulse responses in control solution, with 4 mM Sr^{2+} added and with Sr^{2+} and CTZ (50 μM) added (average of five trials for each condition, 5 ms ISI). Note that Sr^{2+} decreases the first EPSC amplitude and also increases PPD and that CTZ blocks Sr^{2+}-enhanced PPD without markedly increasing the first EPSC amplitude. Open circles show fitted exponential function used to determine the baseline current for measuring the amplitude of the second EPSC.

(B) Mean effects of Sr^{2+}, Sr^{2+} and CTZ, and CTZ alone on the first EPSC amplitude (normalized to control value of 1) and PPD at 5 ms ISI. Sr^{2+} decreases the first EPSC amplitude (n = 9) but increases PPD (n = 5), while CTZ overcomes PPD both in control solutions (n = 5) and when Sr^{2+} is present (n = 6).

(C) Average time course of PPD with Sr^{2+} (open circles, n = 3) shows enhanced PPD at short ISIs, a slower recovery, and a lower plateau level; CTZ with Sr^{2+} (open squares, n = 4) blocks the early part of PPD, but not the plateau phase. Control PPD is shown for comparison (filled circles, n = 30–24 for each point). PPD in control and with Sr^{2+} are fitted with single exponential function (see text), PPD with Sr^{2+} and CTZ is fitted with a linear regression line.

This did not seem to be the case, since the slowing of PPD recovery to plateau levels in the presence of Sr^{2+} was due to lengthening of the period of CTZ-sensitive PPD. Addition of CTZ (50 μM) in the presence of Sr^{2+} abolished PPD at ISIs from 5 to 80 ms (mean EPSC ratio at 5 ms ISI = 0.65 ± 11, n = 6, p = 0.031, compared with Sr^{2+}, paired t test) but had no effect on PPD at ISIs where the EPSC ratio had reached plateau levels (Figures 2B and 2C).

Spontaneous Miniature AMPA EPSCs during Delayed Release following Evoked AMPA EPSCs Do Not Show Amplitude Depression Consistent with Postsynaptic Receptor Desensitization

At synapses, CTZ may not act exclusively to relieve desensitization, since CTZ increases spontaneous miniature EPSC (mEPSC) frequency at the endbulb and other central synapses (Diamond and Jahr, 1995; Isaacson and Walmsley, 1996), an effect usually associated with a presynaptic alteration of release probability (Katz, 1969; Zucker, 1989). We therefore sought to measure directly postsynaptic AMPA receptor desensitization, by examining the amplitude of mESPCs during the period of enhanced mEPSC frequency (delayed release, DR) following an evoked response (Goda and Stevens, 1994; Abdul-Ghani et al., 1996; Otis et al., 1996b).

Delayed release could often be seen under our normal recording conditions (e.g., Figure 3A). In seven neurons, mean peak mEPSC frequency at 10–20 ms after the evoked EPSC was 9.4 ± 4.5 (Figure 3B) times greater than the mean baseline frequency of 5.3 ± 1.6 Hz (Figure 3C) and decayed back to baseline with a time constant of 17.8 ± 1.4 ms (Figure 3B, single exponential fit). However, despite this significant elevation of mEPSC frequency during the period in which PPD was greatest, there was no depression of mEPSC amplitude following the evoked EPSC (Figure 3D). After normalization to mean baseline mEPSC amplitude (Figure 3E), the relationship between mean DR mEPSC amplitude against latency after the evoked EPSC was linear (p = 0.41, runs test) with a slope (0.0008 ± 0.0004) not significantly different from zero (p = 0.06, F test). Mean normalized amplitude for mEPSCs occurring 2–10 ms after the evoked EPSC (0.87 ± 0.05) was not significantly depressed (p = 0.06, one sample t test). As mean PPD at 5 ms ISI was 0.44 in the same conditions, this result suggests that postsynaptic AMPA receptor desensitization does not play a large role in PPD.

It is possible that mEPSC amplitude during DR in control conditions did not show substantial depression because insufficient mEPSCs were collected to detect desensitization at a proportion of the large population of postsynaptic densities present at the endbulb-bushy cell synapse. As illustrated in Figure 3A, we therefore increased DR by addition of Sr^{2+} (4 or 10 mM), with the additional effect of enhancing and prolonging PPD (see above), to maximize the detection of desensitization. Sr^{2+} almost doubled mean peak mEPSC frequency during DR, to 15.8 ± 6.6 times greater (Figure 3B) than baseline frequency in the presence of Sr^{2+} (Figure 3C, 8.3 ± 2.4 Hz, n = 7); it also markedly slowed the decay of mEPSC frequency back to baseline with a time constant of 81.8 ± 6.1 ms (Figure 3B, single exponential fit). Despite this large increase in mEPSC frequency during DR, there was little mEPSC amplitude depression following the evoked EPSC in the presence of Sr^{2+} (Figure 3D). After normalization to mean baseline mEPSC amplitude in Sr^{2+} (Figure 3E), the relationship between mean DR mEPSC amplitude against latency after the evoked EPSC was linear (p = 0.06, runs test) with a slight slope (Figure 3D, 0.0005 ± 0.0002), significantly different from zero (p = 0.001, F test). However, mean normalized amplitude for mEPSCs occurring 2–10 ms after the evoked EPSC (1.03 ± 0.06) was not significantly depressed (p = 0.6, one sample t test). In view of the

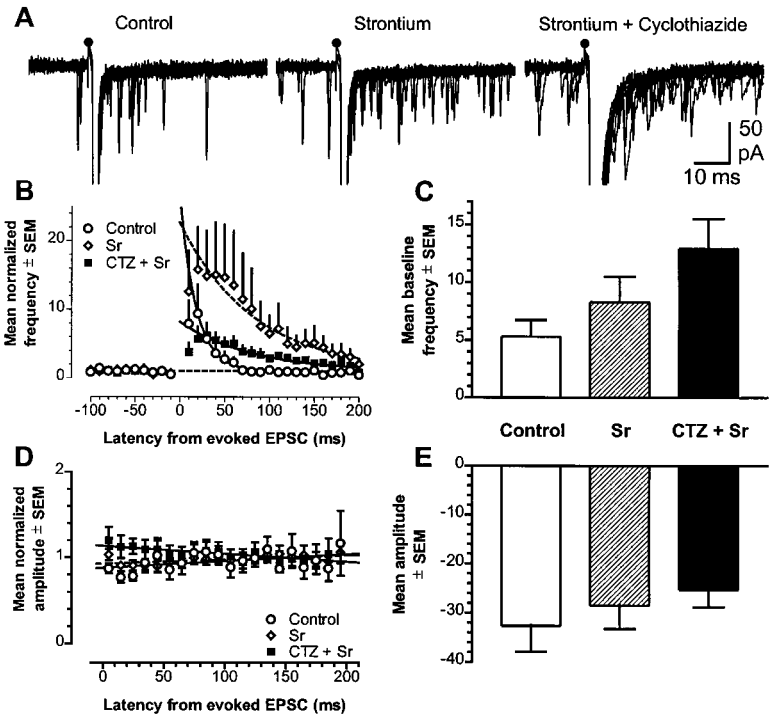

Figure 3. Mean Effects of Sr^{2+} and CTZ on Spontaneous EPSCs following an Evoked EPSC

(A) Examples of spontaneous EPSCs before and after evoked EPSCs (stimulus marked with a closed circle, evoked EPSC is truncated). Each panel contains ten trials in control conditions, with Sr^{2+} added and with Sr^{2+} and CTZ added. Note that spontaneous EPSC amplitude is similar before and after evoked EPSC for each condition.

(B) Mean time course of delayed release of spontaneous EPSCs, normalized to baseline spontaneous EPSC frequency and binned into 10 ms intervals relative to the peak of the evoked EPSC, for control (open circles), with Sr^{2+} (4–10 mM) added (open diamonds), and for Sr^{2+} and CTZ (closed squares, $n = 7$ for all conditions). Each data set during DR is fitted with an exponential function (see text), showing that DR frequency returns to baseline slower in the presence of Sr^{2+}, and this slow return is similar when CTZ is added to Sr^{2+}.

(C) Mean baseline frequency of spontaneous EPSCs measured over 200 ms prior to evoked EPSC, showing the significant increase in spontaneous EPSC frequency with addition of Sr^{2+} and with addition of CTZ and Sr^{2+}.

(D) Mean spontaneous EPSC amplitude during DR is not related to latency after the evoked EPSC. Spontaneous EPSC amplitude has been normalized to the mean baseline spontaneous EPSC amplitude for each cell in each condition and then averaged for all EPSCs occurring in 10 ms bins following the evoked EPSC. The line fitted by linear regression of each data set is shown (solid for control and for CTZ + Sr^{2+}, dashed for Sr^{2+}).

(E) Mean baseline amplitude of spontaneous ESPCs (recorded from 200 ms to 20 ms prior to evoked EPSCs).

enhancement of PPD at a 5 ms ISI in Sr^{2+} (to 0.31, see above), the lack of substantial mEPSC amplitude depression following evoked EPSCs suggests that postsynaptic desensitization does not play a major role in PPD.

Finally, we examined the effects of CTZ on mEPSC frequency and amplitude during DR enhanced by Sr^{2+} (Figure 3A). Addition of CTZ (50–100 μM) in the presence of Sr^{2+} markedly reduced mean DR peak frequency compared with Sr^{2+} alone, to 6.2 ± 1.3 times baseline frequency (Figure 3B), although baseline frequency was further elevated (Figure 3C, 12.9 ± 2.8 Hz, $n = 7$). The decay time constant of mEPSC frequency during DR (79.4 ± 9.6 ms, single exponential fit) was not altered by CTZ (Figure 3B). CTZ thus raised baseline mEPSC frequency compared with Sr^{2+} alone and depressed mEPSC frequency during DR, changes commonly associated with presynaptic alteration of release probability (Zucker, 1989). Although CTZ acted to abolish Sr^{2+}-enhanced PPD over ISIs up to approximately 80 ms, mEPSC amplitude during DR over the same period following an evoked EPSC showed little change. After normalization to mean baseline mEPSC amplitude in CTZ + Sr^{2+} (Figure 3E), the relationship between mean DR mEPSC amplitude against latency after the evoked EPSC was linear ($p = 0.32$, runs test) with a slight slope (Figure 3D, −0.0009 ± 0.0002), significantly different from zero ($p < 0.0001$, F test). However, mean normalized amplitude for mEPSCs occurring 2–10 ms after the evoked EPSC (1.2 ± 0.14) was not significantly increased in comparison to a theoretical mean of 1 ($p = 0.32$, one sample t test) or to the mean mEPSC amplitude for the same bin in Sr^{2+} ($p = 0.15$, paired t test). Thus, with Sr^{2+} present, CTZ decreased PPD from 0.31 to 0.65, a reduction of approximately 50%. For postsynaptic desensitization to account for such a change in PPD, mEPSC amplitude during the initial part of DR should be approximately doubled by CTZ and should decline with an exponential time course. The small and transient increase in mEPSC amplitude present may represent a small remaining bias toward detecting larger amplitude mEPSCs (see Experimental Procedures), detection of summated mEPSCs during the period when DR is highest against the high basal level of spontaneous mEPSC release in CTZ, or a minor contribution of postsynaptic desensitization.

Both AMPA and NMDA Components of Evoked EPSCs Show Paired Pulse Depression

These results suggest that, despite the high quantal content of evoked EPSCs at the endbulb synapse, PPD is not associated with postsynaptic desensitization of AMPA receptors, but rather is due to a decrease in the amount of glutamate released by the presynaptic terminal. Conditions that reduce the amount of presynaptic glutamate release cause a similar decrease in AMPA and NMDA receptor–mediated components of the EPSC (Perkel and Nicoll, 1993; Tong and Jahr, 1994; Von Gersdorff et al., 1997). We therefore sought to confirm this by comparing paired pulse responses of AMPA and NMDA receptor–mediated components of the evoked EPSC at positive membrane potentials (Isaacson and Walmsley, 1995b).

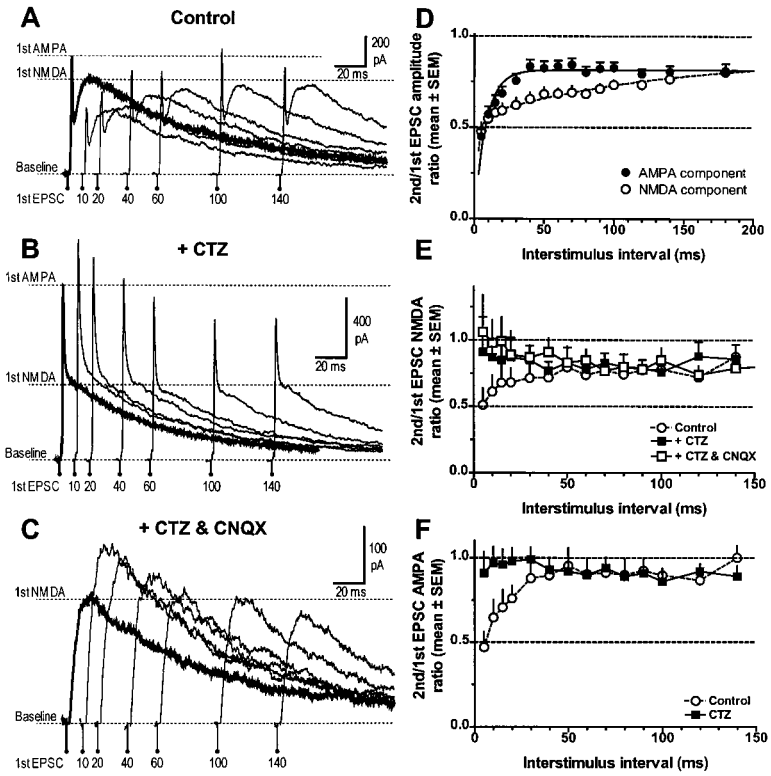

Figure 4. AMPA and NMDA Receptor-Mediated Components of Evoked EPSCs Show CTZ-Sensitive PPD

(A) An example of the marked depression of AMPA and NMDA components of the second evoked EPSC at +50 mV. The response to a single stimulus (thick line, marked as first EPSC) shows a larger, earlier peak due to activation of AMPA receptors and a smaller, later peak due to activation of NMDA receptors. When a second stimulus is given at an ISI between 10 and 140 ms (shown by numbers), both the fast and slow peaks show marked depression. In (A)–(C), underlying currents due to the first EPSC have already been subtracted from the second EPSC, to directly show the EPSC evoked by the second stimulus (see Experimental Procedures). The peak amplitude of the AMPA and NMDA components of the first ESPC are shown with dashed lines. All traces in (A)–(C) are from the same neuron and are averages of five trials.

(B) Addition of CTZ (50 μM) relieves PPD of AMPA and NMDA components and slows the decay of the AMPA component.

(C) CTZ also abolishes PPD of the NMDA component measured in isolation after application of the AMPA receptor antagonist CNQX (20 μM).

(D) Mean PPD of AMPA and NMDA components of evoked EPSCs (n = 29–24 per data point). Both sets of data have been fitted with an exponential function (see text).

(E) Comparison of the mean paired pulse ratio of the NMDA component in control solution, with CTZ, and with CTZ and CNQX (n = 5 for each condition).

(F) Comparison of the mean paired pulse ratio of the AMPA component in control solution and with CTZ (n = 5 for each condition).

Figure 4A shows an example of paired pulse responses at a holding potential of +50 mV, after the subtraction of underlying synaptic current from the first EPSC. Paired stimuli produced PPD of the AMPA component of the evoked EPSC (Figure 4D) that was similar to that seen at −70 mV. The mean ratio of the second to the first EPSC AMPA amplitude at an ISI of 5 ms was 0.45 ± 0.04 (Figure 4D, n = 29), and PPD decreased to a plateau of 0.81 ± 0.01 with a time constant of 8.3 ± 0.6 ms (Figure 4D, single exponential fit).

The NMDA component of the evoked EPSC also showed PPD that was similar to that of the AMPA component at short ISIs, as illustrated in Figures 4A and 4D. Mean NMDA PPD at an ISI of 5 ms was 0.48 ± 0.05, not significantly different from AMPA PPD at the same ISI (p = 0.14, paired t test, n = 29). At longer ISIs, NMDA PPD was variable from cell to cell; 13 of 29 cells recorded showed parallel recovery of AMPA and NMDA PPD, while the remainder showed NMDA PPD that was always greater than AMPA PPD for the same ISI. Due to this enhanced NMDA PPD at longer ISIs in some cells, the time course of mean NMDA PPD for all cells was best fitted by a double exponential function with time constants of 2.8 ± 0.4 ms (62%) and 130 ± 47.9 ms (38%) (Figure 4D).

If postsynaptic desensitization of AMPA receptors is the primary mechanism underlying PPD, then there should be a marked difference in PPD of AMPA and NMDA components of the EPSC at short ISIs. Our observation that the AMPA and NMDA receptor–mediated components of evoked EPSCs show similar PPD at short ISIs strongly suggests that there is a presynaptically regulated reduction of glutamate release in response to the second stimulus (Perkel and Nicoll, 1993). The marked PPD of the NMDA component of evoked EPSCs is unlikely to be principally due to NMDA receptor desensitization for a number of reasons. NMDA receptors do not show substantial desensitization over the relatively short time scales (<50–80 ms) involved in CTZ-sensitive PPD at this synapse (McBain and Mayer, 1994). Calcium-dependent NMDA receptor desensitization (Legendre et al., 1993) is unlikely to be involved, since 10 mM EGTA was included in the patch pipette solution, and Ca^{2+} influx should be negligible at the positive holding potential used. Glycine-sensitive desensitization (Benveniste et al., 1990) is also unlikely, since saturating concentrations of glycine appear to be present in the cochlear nucleus slice (Isaacson and Walmsley, 1995b). While some cells showed greater PPD of the NMDA component than of the AMPA component at longer ISIs, the fact that NMDA PPD was similar at short ISIs for all cells and closely paralleled AMPA PPD in a substantial number of cases suggests that an additional mechanism may contribute to enhanced NMDA PPD at longer ISIs.

Cyclothiazide Blocks Paired Pulse Depression of AMPA and NMDA Components of Evoked EPSCs

The similar reduction of AMPA and NMDA EPSCs provides a further test for the site of action of CTZ on PPD. Since CTZ does not alter responses of postsynaptic

NMDA receptors (Hoyt et al., 1995), CTZ should abolish PPD of both AMPA and NMDA components, if the site of action of CTZ is presynaptic, but would only abolish AMPA receptor–mediated PPD if CTZ acts postsynaptically to block desensitization.

As illustrated in Figure 4B, we found that CTZ (50 μM) abolished PPD of both AMPA and NMDA components. In the presence of CTZ, the mean AMPA and NMDA EPSC ratios at an ISI of 5 ms were 0.88 ± 0.11 and 0.71 ± 0.09, respectively (Figures 4E and 4F, n = 5 cells). This change in NMDA EPSC ratio was not due to broadening of the AMPA component to contaminate measurements of the NMDA receptor–mediated current, as adding CNQX (20 μM) to block AMPA receptors did not decrease the effect of CTZ on NMDA PPD (Figure 4C). Mean NMDA EPSC ratio at an ISI of 5 ms with CTZ and CNQX present was 1.06 ± 0.31 (Figure 4F, n = 5). The sensitivity of NMDA component PPD to CTZ also suggests that depression of the NMDA component is not primarily due to postsynaptic saturation of NMDA receptors (Lester et al., 1990), since CTZ was able to increase markedly the amplitude of the NMDA component.

The ability of CTZ to abolish NMDA receptor–mediated PPD provides compelling evidence that PPD at the endbulb is due to a presynaptic mechanism that markedly decreases synaptic release in response to a second stimulus, when a prior stimulus has occurred within the last 50–80 ms, and suggests this presynaptic mechanism is CTZ-sensitive. This transient decrease in release probability could be due to several presynaptic mechanisms, such as depletion of the releasable pool of synaptic vesicles, alteration of the presynaptic action potential or other effects of presynaptic calcium influx. The following experiments were designed to distinguish between these alternative mechanisms.

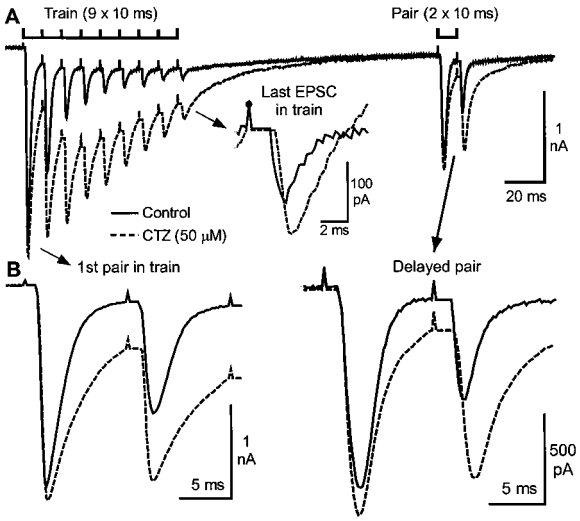

Figure 5. Depletion of Synaptic Vesicles Does Not Alter PPD

(A) Depletion of vesicle release does not reduce PPD and is insensitive to CTZ. A train of nine stimuli at 100 Hz depletes the pool of releasable vesicles, reducing synaptic release to approximately 10% of initial release levels in control solution (unbroken traces). After a delay of 125 ms, synaptic release in response to the first stimulus of a pair has recovered to approximately 50% of initial levels, but PPD is similar (see [B]). CTZ (50 μM, dashed traces) does not block depletion by the end of the stimulus train; an inset shows the last EPSC in the train with equal baselines for control and CTZ.

(B) CTZ is equally effective at reducing PPD before and after the depleting stimulus train. The first pair of EPSCs in the train of stimuli and the paired stimuli after a delay, shown at expanded time scale, and with amplitude scaled so that the peak of the first EPSC is equal.

Depletion of Releasable Vesicles Does Not Alter Cyclothiazide-Sensitive Paired Pulse Depression

Synaptic depression at other synapses has been attributed to depletion of a pool of release-competent synaptic vesicles (Liley and North, 1953; Thies, 1965; Kusano and Landau, 1975; Stevens and Wang, 1995; Dobrunz and Stevens, 1997; Von Gersdorff et al., 1997; Dittman and Regehr, 1998; Wang and Kaczmarek, 1998). If this is the case at the endbulb, depletion of releasable vesicles should alter PPD and should be CTZ-sensitive. At the calyx of Held, a morphologically and functionally similar auditory synapse in the medial nucleus of the trapezoid body, it has been shown that a train of stimuli at high frequency markedly reduces synaptic release by depleting the releasable pool of vesicles (Wang and Kaczmarek, 1998). We therefore used a similar train of stimuli to investigate the effects of depletion on PPD and to determine whether CTZ had differential effects on PPD and vesicle depletion.

In 11 cells, trains of five to ten stimuli at 100 Hz were given, causing marked reduction of evoked EPSCs (e.g., Figure 5); the mean amplitude of the last EPSC in the train was 7.5% ± 1.3% of the amplitude of the first EPSC in the train. This stimulation pattern reduced synaptic release to a steady state value for successive stimuli; the mean amplitude ratio of the last two EPSCs in the train was 0.92 ± 0.10 (p = 0.45 compared with theoretical mean of 1.0, one sample t test). As conditions at the synapse at the end of the stimulus train will be markedly different from those resulting from a single pair of stimuli, a delay of 75–150 ms following the end of the train was interposed, to allow cumulative effects of the stimulus train to dissipate before a pair of stimuli at 10 ms separation were given. This delay was timed for each cell to allow the evoked EPSC to partially recover to a similar degree across all cells; the mean amplitude of the first EPSC in the delayed pair was 49% ± 2% of the first EPSC in the train. Despite the substantial remaining depletion, PPD for the delayed pair of EPSCs (0.49 ± 0.08) was not significantly different from PPD for the first pair of EPSCs in the train (0.43 ± 0.06). As the number of vesicles released in the first delayed EPSC has been reduced by half following depletion and partial recovery, the lack of a similar reduction in PPD of the delayed pair of EPSCs suggests that PPD may not be due to depletion and also provides further evidence against postsynaptic desensitization.

If depletion and CTZ-sensitive PPD are due to separate mechanisms, CTZ should be ineffective at blocking depletion, but equally effective at reducing PPD in the control and depleted condition. In the presence of CTZ (50 μM, n = 4 cells), a stimuli train resulted in a mean amplitude of the last EPSC of 8.3% ± 1.3% of the first EPSC, indicating that CTZ was not effective at blocking vesicle depletion. However, CTZ was effective at reducing PPD for both the first pair of EPSCs in the train (0.65 ± 0.17, p = 0.038, paired t test compared with control PPD) and the delayed pair of EPSCs (0.74 ±

0.18, p = 0.001, paired t test compared with control PPD). These results suggest that, at the endbulb, PPD and vesicle depletion are regulated by different mechanisms, which can be distinguished by their sensitivity to CTZ.

Presynaptic Calcium Influx Regulates Cyclothiazide-Sensitive Paired Pulse Depression

As PPD appeared to be relatively independent of vesicle depletion, we determined the effects of altering external Ca^{2+} levels on PPD, to ascertain whether PPD might be related to effects of Ca^{2+} influx during the first stimulus, which altered release probability in response to the second stimulus, or was due to Ca^{2+}-independent changes in release probability, such as alteration of steps in vesicle release downstream from Ca^{2+} binding to the exocytotic Ca^{2+} sensor.

At other synapses showing PPD, raising external Ca^{2+} increases transmitter release during the first EPSC and enhances PPD (Dittman and Regehr, 1998), a response compatible with either an increase in presynaptic depletion or in postsynaptic desensitization or enhancement of a calcium-sensitive presynaptic mechanism. Interestingly, at the endbulb, raising external Ca^{2+} from 2 mM (normal Ca^{2+}) to 3 mM (high Ca^{2+}) did not cause significant increases in the first EPSC amplitude (Figure 6A); the mean amplitude of the first EPSC was increased by 9.7% ± 5.9% (n = 3, p = 0.24, compared with theoretical mean of 100%, one sample t test). However, in high Ca^{2+}, PPD at an ISI of 5 ms was significantly greater; the mean PPD of AMPA-mediated EPSCs was lowered from 0.43 ± 0.08 to 0.28 ± 0.08 (Figure 6B; p = 0.043, n = 3, paired t test). As synaptic release during the first EPSC was not increased in high Ca^{2+}, these results provide further evidence against both postsynaptic desensitization and presynaptic vesicle depletion as the mechanism causing PPD at the endbulb and suggest that increasing presynaptic Ca^{2+} influx during the first EPSC enhances PPD.

If presynaptic Ca^{2+} influx regulates PPD, then reduction of Ca^{2+} influx should reduce or even abolish PPD. However, since decreasing presynaptic Ca^{2+} influx typically decreases transmitter release, such a response is also equally compatible with a decrease in presynaptic depletion or in postsynaptic desensitization. In order to distinguish between these alternatives, we compared paired pulse responses seen after lowering external Ca^{2+} to 1 mM (low Ca^{2+}) with paired pulse responses during partial depletion. The value of this comparison is that, while the level of release in the two conditions is similar (mean first EPSC amplitude was 62% ± 7% of control in low Ca^{2+} compared with 49% of control following depletion), presynaptic Ca^{2+} influx will be reduced in low Ca^{2+} but maintained at normal levels during partial depletion. In marked contrast to the failure of partial depletion to alter PPD (see above), low Ca^{2+} abolished PPD (Figure 6C). The mean amplitude ratio of AMPA-mediated ESPCs at an ISI of 5 ms was significantly increased from 0.44 ± 0.07 in control Ca^{2+} to 0.97 ± 0.20 in low Ca^{2+} (Figure 6B; p = 0.014, n = 6, paired t test).

Together, these results clearly demonstrate that the degree of PPD is highly dependent on the level of Ca^{2+} influx into the endbulb in response to the first stimulus, and not on the number of vesicles or amount of glutamate released in the first EPSC, providing further evidence against both presynaptic depletion and postsynaptic desensitization as major sources of PPD.

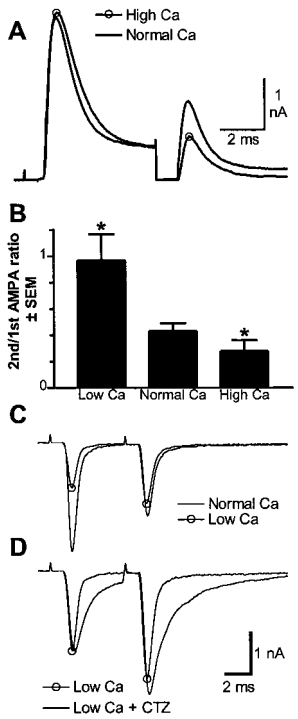

Figure 6. Paired Pulse Responses Are Determined by the Level of Presynaptic Calcium Influx

(A) Raising extracellular Ca^{2+} concentration from 2 mM (normal Ca^{2+}) to 3 mM (high Ca^{2+}) enhances PPD without increasing the number of vesicles released in the first EPSC. The peak amplitude of the AMPA component of the first and second EPSCs in high Ca^{2+} are marked with open circles. EPSCs recorded at +50 mV; the residual current of the first EPSC has been subtracted from the second EPSC. In this and other panels, each trace is an average of five trials in each condition.

(B) Mean PPD of the AMPA EPSC in low Ca^{2+} (n = 5, 1 mM external Ca^{2+}), normal Ca^{2+} (n = 8), and high Ca^{2+} (n = 3).

(C) Lowering external Ca^{2+} concentration to 1 mM (low Ca^{2+}) alleviates AMPA EPSC PPD. In (C) and (D), peak amplitude of the first and second EPSCs (recorded at −70 mV) in low Ca^{2+} are marked with open circles.

(D) Lowering external Ca^{2+} concentration occludes the effect of CTZ on paired pulse responses. Paired pulse facilitation of AMPA EPSCs in 1 mM external Ca^{2+} (low Ca^{2+}) is little changed by the addition of CTZ (50 μM). The traces in low Ca^{2+} have been scaled, so that the first EPSC amplitude equals that of the first EPSC in low Ca^{2+} + CTZ. Calibration shown in the panel refers to both traces in (C) and the low Ca^{2+} + CTZ trace in (D); in (D), current calibration in low Ca^{2+} is 1.76 nA.

Finally, since either low Ca^{2+} or CTZ alleviates PPD, if both conditions operate via a common mechanism, these conditions should at least partially occlude each other. This is the case, as illustrated in Figure 6D; in four cells recorded in low Ca^{2+}, addition of CTZ (50 μM) caused only a small increase in the paired pulse ratio of the second EPSC relative to the first EPSC (+38% ± 14%, compared with paired pulse ratio in low Ca^{2+}). This mean change in paired pulse ratio on addition of CTZ in low Ca^{2+} was significantly less (p = 0.049) than that

seen when CTZ was added in normal Ca^{2+} (+125% ± 36%, n = 9). These results thus suggest that decreasing the level of Ca^{2+} influx in response to the first EPSC produces a similar effect to that of CTZ and that these two conditions act via a common mechanism.

Discussion

Fast, secure synaptic transmission at the endbulb-bushy cell synapse is essential for sound localization (Oertel, 1997; Trussell, 1997). This is achieved by single fiber EPSCs whose large amplitude and brief duration ensure synchrony between presynaptic and postsynaptic action potentials. In the chick brainstem, the calyceal synapse between nucleus magnocellularis neurons and cochlear nerve fibers plays an analogous role and shows marked PPD at least partly due to postsynaptic AMPA receptor desensitization (Trussell et al., 1993; Otis et al., 1996b). In contrast, our results provide evidence for a novel mechanism causing PPD at the rat endbulb synapse, involving a transient Ca^{2+}-dependent and CTZ-sensitive presynaptic decrease in transmitter release.

AMPA Receptor Desensitization and Synaptic Depression

CTZ blocks desensitization of AMPA receptors in excised membrane patches (Westbrook and Jones, 1996), although it also alters AMPA receptor-gated glutamate channel kinetics (Patneau et al., 1993; Yamada and Tang, 1993). At the chick magnocellularis synapse, AMPA receptors in membrane patches show rapid desensitization with a time course similar to that of the decay of evoked EPSCs in brain slices, while recovery from desensitization parallels recovery of evoked EPSCs from PPD (Trussell, 1997). As CTZ blocked membrane patch desensitization, slowed the evoked EPSC decay, and reduced PPD at the chick synapse, it was proposed that AMPA receptor desensitization contributed significantly to shaping the EPSC decay and to PPD (Trussell et al., 1993; Otis et al., 1996b). At the rat endbulb-bushy cell synapse, CTZ also slows evoked EPSC or mEPSC decay and blocks PPD (Isaacson and Walmsley, 1996). However, at the endbulb, CTZ-induced changes in evoked EPSC shape are due to the slower decay of quantal mEPSCs, since the evoked EPSC in CTZ can be reproduced by convolution of the quantal mEPSC in CTZ with the probability of quantal release (Isaacson and Walmsley, 1995a; Isaacson and Walmsley, 1996). Thus, if quantal content remains constant, AMPA receptor desensitization will not decrease the evoked EPSC unless it also substantially decreases quantal amplitude. In contrast to the substantial mEPSC amplitude depression seen at the chick magnocellularis synapse (Otis et al., 1996b), in the present study of the endbulb synapse, we found that mEPSC amplitude during normal and Sr^{2+}-enhanced DR showed little time-dependent depression, suggesting that postsynaptic desensitization of AMPA receptors is unlikely to play a major role in PPD at the rat endbulb synapse.

Presynaptic Effects of Cyclothiazide

Application of CTZ to brain slices or cultures clearly has effects on synaptic transmission difficult to equate with a purely postsynaptic site of action. CTZ increases mEPSC frequency at the endbulb (Isaacson and Walmsley, 1996) and other synapses (Yamada and Tang, 1993; Diamond and Jahr, 1995; Mennerick and Zorumski, 1995), an effect due to a presynaptic change in release probability (Katz, 1969). We have also now shown that CTZ can alter other presynaptic forms of short-term synaptic plasticity, such as the delayed release of mEPSCs following evoked transmission. Finally, our finding that CTZ blocks PPD of both AMPA and NMDA components of evoked EPSCs at the endbulb-bushy cell synapse provides compelling evidence for a presynaptic action of CTZ.

Depletion as a Presynaptic Mechanism for Synaptic Depression

At different synapses, paired pulse depression may be due to different mechanisms—postsynaptic receptor desensitization, presynaptic depletion of release-competent vesicles, or other presynaptic factors transiently depressing vesicle release probability. At the endbulb, our data suggests that a CTZ-sensitive presynaptic mechanism is the prime determinant of PPD over short ISIs and that postsynaptic desensitization does not play a major role in PPD.

At several central and peripheral synapses, a common mechanism of synaptic depression is depletion of a pool of release-competent synaptic vesicles, thus lowering the quantal content of following responses until this pool has been replenished (Liley and North, 1953; Thies, 1965; Kusano and Landau, 1975; Stevens and Wang, 1995; Dobrunz and Stevens, 1997; Von Gersdorff et al., 1997; Dittman and Regehr, 1998; Wang and Kaczmarek, 1998). However, our results are not consistent with depletion as the major mechanism underlying PPD at the endbulb synapse. We found that approximately 50% depletion did not alter PPD and that CTZ did not block depletion (Wang and Kaczmarek, 1998) but did block PPD in the depleted state. These results directly demonstrate that depletion and paired pulse depression are due to two separate mechanisms, depletion being insensitive, and depression sensitive, to CTZ. Other results also suggest that PPD at the endbulb synapse is not due to vesicle depletion. For example, addition of strontium reduced the number of vesicles released in the first EPSC, yet markedly enhanced PPD, while high external Ca^{2+} significantly increased PPD without substantially increasing vesicle release in the first EPSC.

Indeed, it is unlikely that a single stimulus will substantially reduce the pool of available vesicles at the endbulb. Serial electron micrography of the endbulb reveals at least several hundred release sites per endbulb, each with a population of morphologically "docked" vesicles (M. J. Nicol and B. W., unpublished data), while a single presynaptic action potential releases only 30–100 quanta (Bellingham et al., 1998). The failure of depletion to alter PPD is thus consistent with this large mismatch.

Calcium Influx as a Presynaptic Factor in Synaptic Depression

Several forms of short-term synaptic plasticity, including posttetanic facilitation, paired-pulse facilitation and depression, and enhanced frequency of spontaneous release

following evoked release (so-called delayed release) are mechanistically different from Ca^{2+}-dependent triggering of evoked release, as they require lower concentrations of Ca^{2+}, are reduced by slow chelators of free Ca^{2+}, and are supported by Sr^{2+} and other divalent ions (Zucker, 1989; Van der Kloot and Molgó, 1994). This difference has led to the postulation of two distinct Ca^{2+} binding sites in the presynaptic terminal—a low-affinity site, responsible for triggering evoked release, and one or more higher-affinity sites that bind Ca^{2+}, Sr^{2+}, and other divalent cations, responsible for various forms of short-term plasticity (Goda and Stevens, 1994; Zucker, 1996). The addition of Sr^{2+} significantly enhanced and prolonged both CTZ-sensitive PPD and DR at the endbulb, suggesting that both of these forms of short-term plasticity may be modulated by a Sr^{2+}-sensitive presynaptic mechanism acting via the high-affinity site for Ca^{2+}.

Increasing Ca^{2+} influx into the endbulb increased PPD, while decreasing Ca^{2+} influx decreased PPD and occluded the effects of CTZ on PPD. It is unlikely that CTZ acts by inhibiting presynaptic Ca^{2+} channels, since this would also substantially reduce the first EPSC. Indeed, presynaptic Ca^{2+} tail currents in the calyx of Held show slight enhancement at ISIs of 5–10 ms (Forsythe et al., 1998; Wang and Kaczmarek, 1998). In addition, CTZ increases mEPSC frequency independent of presynaptic calcium entry at the endbulb and other synapses (Diamond and Jahr, 1995; Isaacson and Walmsley, 1996), indicating that it can modulate release probability without altering Ca^{2+} influx. Thus, the simplest interpretation of the changes in PPD at the endbulb induced by altering external Ca^{2+} concentration is that sufficient Ca^{2+} entering the terminal can activate a process that transiently inhibits vesicle release in response to a subsequent action potential, while CTZ acts as an antagonist for activation of this process. To our knowledge, this constitutes a novel mechanism of synaptic depression at a central synapse, which is possibly similar to the Ca^{2+}-dependent adaptation of transmitter release observed in the squid giant axon presynaptic terminal (Hsu et al., 1996).

Calcium entry following an action potential thus seems to transiently regulate future release via a high-affinity site, whose occupation either activates some Ca^{2+}-dependent production of a second messenger regulating exocytosis or provides a buffer that releases Ca^{2+} relatively slowly (Yamada and Zucker, 1992; Winslow et al., 1994; Neher, 1998). Ca^{2+}-dependent PPD may be particularly potent at a multirelease site synapse such as the endbulb, as localized intraterminal changes in Ca^{2+} concentration due to Ca^{2+} influx will equilibrate rapidly to much lower but elevated levels throughout calyceal terminals (Helmchen et al., 1997), influencing release probability at sites distant to those that released previously. It will be important to quantitate the time course and concentration of intraterminal Ca^{2+} in the endbulb to determine the relationship between PPD and Ca^{2+} levels.

It is thought that a presynaptic action potential will usually cause release of, at most, a single synaptic vesicle at an individual release site (Stevens and Wang, 1995), despite morphological evidence showing that many vesicles are docked at presynaptic release zones (Harris and Sultan, 1995) and estimates that up to a dozen synaptic vesicles may be release-competent per site (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). The process restricting release to a single vesicle apparently persists for an appreciable time, acting as a "brake" to transiently decrease the probability that Ca^{2+} influx will trigger vesicle release at the same site (Stevens and Wang, 1994). This transient reduction in release probability is consistent with a marked decrease in quantal content of evoked EPSCs, following prior evoked release, and we speculate that it may be similar to the Ca^{2+}-dependent mechanism described here.

A Calcium-Dependent Molecular Mechanism for Synaptic Depression?

Ultimately, the molecular mechanism for depression is likely to involve a Ca^{2+}-dependent reduction in the functions of one of the presynaptic proteins involved in controlling vesicle release. As an example, interactions between Ca^{2+} channel proteins and syntaxin, SNAP-25 and synaptotagmin (Kim and Catterall, 1997; Chapman et al., 1995), translocation of synaptotagmin to the cell membrane (Chapman and Jahn, 1994), and triggering of synaptotagmin dimerization (Chapman et al., 1996) can be Ca^{2+} dependent and therefore could be transiently affected by Ca^{2+} entry. In *Drosophila* and mice, genetic reduction of synaptotagmin, the presumed Ca^{2+} sensor for synaptic release, decreases evoked transmission, raises spontaneous vesicle release, and decreases the number of docked vesicles (DiAntonio and Schwarz, 1994; Geppert et al., 1994; Reist et al., 1998). A transient Ca^{2+}-dependent reduction in synaptotagmin function following vesicle release could thus account for both reduction of evoked release and enhanced rates of spontaneous release following the first stimulus. However, genetic manipulation of other synaptic proteins, such as synaptobrevin, NSF, or cysteine string proteins can also reduce synaptic transmission (Wu and Bellen, 1997); their possible roles in PPD and the Ca^{2+} dependence of their functions remain relatively unexplored.

In conclusion, our studies of PPD at the endbulb synapse have revealed that, following nerve-evoked transmitter release, there is an extremely rapid inhibition of subsequent release that is not due to presynaptic vesicle depletion or postsynaptic receptor desensitization. This inhibitory mechanism is governed by the level of presynaptic Ca^{2+} influx, most probably due to Ca^{2+} binding to a molecular site intimately involved in regulating the probability of synaptic vesicle release.

Experimental Procedures

Slice Preparation and Recording Methods

Recordings (n = 49 cells) were made from superfused parasagittal slices (150 μm thick) of the anteroventral cochlear nucleus from 10- to 17-day-old *Wistar* rats (n = 22) of either sex, prepared as described previously (Isaacson and Walmsley, 1995a, 1995b, 1996; Bellingham et al., 1998). In brief, animals were deeply anesthetized with sodium pentobarbitone (20 mg/kg, intraperitoneally), then killed by decapitation after their responses to noxious stimuli (withdrawal and corneal reflexes) ceased. The brainstem was rapidly removed, and slices were made (DSK Microslicer, Dosaka Instruments, Japan) in ice–cold Ringer as given below, with the exception that $MgCl_2$ and $CaCl_2$ concentrations were 5 and 1 mM, respectively. Slices were held in this solution at 34°C–37°C for 1 hr, then transferred to

a Ringer solution containing (in mM): 119 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, and 20 μM strychnine HCl, equilibrated with 95% O_2/5% CO_2 at room temperature for holding and recording. Patch electrodes (1.5–3 MΩ resistance) contained (in mM): 117.5 Cs−gluconate, 17.5 CsCl, 4 NaCl, 10 HEPES, 3 Mg−ATP, 0.2 Na−GTP, and 10 EGTA (pH 7.3). Series resistance (<10 MΩ) was routinely compensated by >80%. Slices were viewed by video microscopy (C-2400–07 ER video camera, Hamamatsu, Japan) with infrared translumination (Omega Optical, Brattleboro, VA, filter band pass 750−790 nm) and Nomarski optics (Axioskop FS, Carl Zeiss, Goettingen, Germany). EPSCs were evoked with a voltage pulse (0.1 ms, 2–100 V) via a Ringer−filled pipette (tip diameter, 10–20 μm) placed near a branch of the auditory nerve coursing toward the recorded cell. Single fiber inputs had stable EPSC amplitude responses without intermediate levels, no latency variation, stable amplitude over a range of at least 5 V suprathreshold, and all-or-none responses at threshold stimulus levels. EPSCs arising from endbulbs were distinguished by their all−or−none response to stimulation and fast kinetics at −70 mV (Isaacson and Walmsley, 1995a, 1995b, 1996; Bellingham et al., 1998). Synaptic currents were recorded and filtered at 5–10 kHz with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) before being digitized at 20–40 kHz on a 16 bit A-D converter (Digidata 1200A, Axon Instruments). Data were also recorded on video tape with a pulse code modulated video recorder at 44 kHz (A. R. Vetter Instruments, Rebersburg, PA) and digitized later off-line. Experiments were performed at room temperature (22°C–25°C).

All drugs were obtained from Sigma (St. Louis, MO), except for cyclothiazide (CTZ, gift of Eli Lilly, Cincinnati, IL) and 6-cyano-7-nitroquinoxaline (CNQX, Tocris Cookson, Bristol, United Kingdom). Drugs were applied via a gravity-fed line (flow rate, 2–3 ml/min) to a recording chamber with a volume of 0.5 ml for a minimum of 4 min before measurements were made, to allow equilibrium to be reached. CTZ and CNQX were prepared as stock solutions of 50 mM in methanol and 20 mM in dimethyl sulphoxide, respectively, and frozen until use. Solvent concentrations were 0.1% or less in the bathing solution.

Data Analysis

Data were analyzed with the use of pClamp 6 and Axograph 3 (Axon Instruments) and Excel (Microsoft, Redmond, WA). Data are shown as mean ± SEM. The statistical test used was an unpaired two-tailed Student's t test, unless otherwise stated; all curve fitting and statistical tests were done with Prism 2 (Graphpad, Sorrento, CA). Measurements were made on averaged traces (n = 3 or more trials). The amplitude of the fast AMPA component was determined by subtracting the mean baseline current over a period (0.25−1 ms) immediately preceding the event from the peak value. The amplitude of the slow NMDA component of EPSCs was measured as the average current during a 5 ms window centered around the peak of the NMDA component, minus baseline current. For an individual neuron, the placement of this 5 ms window after the stimulus was kept constant for all measurements, except those during the application of CTZ, when the window was placed further along the trace to avoid contamination with the AMPA component. For measurement of the second EPSC at positive holding potentials, the underlying current due to the NMDA component of the first EPSC was first eliminated, by fitting the NMDA component of an EPSC evoked by a single stimulus with a double exponential function, to mimic the time course of the NMDA component, and then subtracting this appropriately scaled function from the response to the paired stimulus.

Spontaneous EPSCs were detected using semi−automated detection procedures in Axograph 3 (Axon Instruments) (Clements and Bekkers, 1997). The amplitude threshold for detection was three times the standard deviation of the baseline noise, and mEPSCs occurring within 0.5–1 ms of each other were excluded from analysis. The decay phase of evoked EPSCs resulted in a period of sloping baseline for a variable period following the peak of the evoked EPSC; this was particularly prominent when CTZ was applied. This sloping baseline resulted in a systematic bias against detection of small amplitude mEPSCs. To minimize this bias, for each trial, the decay of the evoked EPSC current was well fitted with the sum of two or three exponential functions, and the waveform of this function was subtracted from the trial, before mEPSC detection was done. Onset latency of mEPSCs was measured relative to the time of the evoked EPSC peak. Control mEPSCs were detected during the 200 ms prior to the evoked EPSC (excluding 10–20 ms immediately prior to the evoked EPSC), while mEPSCs during the delayed release (DR) phase were detected for a period covering 200 ms after the evoked EPSC (excluding the first 1–3 ms following the evoked EPSC peak). All mEPSCs were baselined to the mean current immediately prior to their onset. Only mEPSCs with 10%–90% rise times of 50–400 μs and half widths of 0.2 to 0.8 or 1.6 (CTZ only) ms were accepted for analysis; these criteria excluded less than 5% of detected events. All accepted mEPSCs were inspected visually prior to final analysis.

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